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A Novel Vpr Peptide Interactor Fused to Integrase (IN) Restores Integration Activity to IN-Defective HIV-1 Virions

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A novel approach to complement human immunodeficiency virus type I (HIV-1) integrase (IN)-defective virions has been identified. The approach involves fusion of a 23-amino-acid stretch to the N-terminus of wild-type IN and coexpression of this chimera with the IN-defective proviral template in virus producing cells. The 23-amino-acid peptide represents a Vpr "interactor," referred to as the the WxxF or WF domain, which apparently leads to docking of the domain along with the fusion partner onto HIV-1 Vpr, thus permitting virion incorporation of the chimeric protein when expressed, *in trans,* with other viral products. Transfection of the WF-IN expression plasmid along with HIV-1 viral clones that produce Vpr, but bear an IN mutation, results in the release of a proportion of viral particles that are competent for integration. The extent of complementation was assessed using the MAGI cell assay, where integration of viral DNA results in the eventual appearance of easily visible multinucleated blue syncytia. The efficiency of dWF-IN (double copy of WF domain) complementation is not improved markedly by incorporation of a HIV-1 protease cleavage site (PR) between the dWF domain and IN (dWF-PR-IN), unlike that observed with Vpr fusions to IN. Furthermore, the ability of Vpr-PR-IN and dWF-PR-IN to complement IN-defective proviral clones, both of which bear an intervening protease cleavage site, appear comparable. Western blotting analyses using virions isolated through sucrose cushions demonstrate clearly the incorporation of the dWF-IN fusion protein into Vpr containing HIV-1 particles but not in Vpr-deficient virions. Additional Western blotting analyses indicate that all Vpr-IN and dWF-IN chimeras, with or without a PR site, are packaged into virions. The efficiency of virion incorporation of Vpr-IN and dWF-IN chimeras appears approximately comparable by Western blotting analysis. The ability of dWF-IN to complement IN-defective proviruses with efficiency similar to that of Vpr-PR-IN and dWF-PR-IN indicates that dWF-IN retains the full complement of functions necessary for integration of proviral DNA and is likely due to the benign nature of this small domain at the amino-terminus of IN.

Key Words: HIV-1; Vpr; integrase; virions.

INTRODUCTION

The efficient replication of retroviruses requires integration of the double-stranded DNA copy of viral RNA into chromosomal DNA of the infected host cell (Kulkosky and Skalka, 1994; Farnet and Bushman, 1996). Integration for all retroviruses, including human immunodeficiency virus type 1 (HIV-1) (La Femina *et al.,* 1992), represents the stable incorporation of viral DNA into that of the host and depends upon the function of the viral-encoded enzyme integrase.

Integration occurs in two discrete steps (Goff, 1992). First, integrase (IN) removes two nucleotides from the 3′ ends of the newly synthesized viral DNA within the cytoplasm of the infected host cell (Roth *et al.,* 1989; Brown *et al.,* 1989). This step is typically referred to as the processing reaction. The processed viral DNA, which is contained within a larger nucleoprotein complex in the infected cell (Bowerman *et al.,* 1989), is then inserted into host chromosomal DNA. This insertion step, which represents a trans-esterification event, is referred to as the joining reaction.

The biochemical mechanisms that underpin IN processing and joining have been elucidated largely by the use of simple *in vitro* assays that require only purified recombinant enzyme and short oligodeoxynucleotide duplex DNA substrates (Katzman *et al.,* 1989; Craigie *et al.,* 1990; Katz *et al.,* 1990; Engleman *et al.,* 1993). Amino acid sequence alignments for several IN species and extensive mutagenesis of the protein have defined conserved functional regions (Engelman *et al.,* 1993), including the active site of the enzyme, which is referred to as the D,D(35)E domain (Kulkosky *et al.,* 1992; Engelman and Craigie, 1992). The recent X-ray crystal structure of the D,D(35)E domain of HIV-1 IN suggests that the design of effective active site inhibitors may soon be feasible (Dyda *et al.,* 1994).

The rapid advances in our understanding of IN structure and function, in conjunction with simple and rapid *in vitro* activity assays, have provided the basis for a new generation of studies directed toward: (1) inhibition of IN

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activity (Levy-Mintz *et al.,* 1996) by rational design; (2) the addition of various discrete DNA binding domains (Bushman 1995; Goulaouic and Chow, 1996; Katz *et al.,* 1996) to convert integration into a site-specific process; or (3) redirection of its mode of delivery into virions (Liu *et al.,* 1997; Fletcher *et al.,* 1997) in order to assess IN's contribution to virion architecture or to define its possible interaction with other virion proteins that may affect the dynamics of the integration process.

With regard to the last efforts, we have identified a novel strategy to deliver IN into virus particles, the mechanism of which is independent of the natural mode of incorporation that involves protease maturation of the Gag-Pol polypeptide precursor. The approach employs an in-frame fusion of a 23-amino-acid stretch to the N-terminus of IN. Interestingly, this 23-amino-acid peptide represents a Vpr "interactor" found in uracil DNA glycosylase (UDG), which is referred to as the WxxF or WF domain (BouHamdan *et al.,* 1998). A double-cassette of the WF motif, designated dWF, fused to the bacterial chloramphenicol acetyl transferase (CAT) gene leads to import of the chimera into HIV-1 virus particles (BouHamdan *et al.,* 1998). Previous studies indicate that fusion to the WF motif double-cassette leads to incorporation of higher levels of the chimera into viral particles (BouHamdan *et al.,* 1998). The dWF-IN chimera is incorporated into virions, likely by docking to the viral accessory protein, Vpr, and can restore integration activity to full-length genomic viral templates that bear a variety of IN mutations. The copy number of chimeric molecules delivered appears comparable to the levels of proviral derived IN (~50 to 150 copies) and is likely related to the ability to be transported by associating with the higher copy number of Vpr molecules that are encapsidated within HIV-1 particles (Cullen, 1998). This strategy for the *in trans* delivery of IN into whole virions, which is independent of the expression of products from the viral genome, could be useful for assessing rapidly the *in vivo* effects of IN mutations to further advance structure/function studies of the protein. The strategy might also be used to define the critical interactions between IN and other viral proteins that contribute to virion architecture. Finally, this mode of delivery could also be exploited as a novel means to direct protein-based inhibitors into mature HIV-1 particles aimed at reducing or eliminating virion infectivity.

RESULTS

Syncytia-forming assay to assess dWF-IN complementation

Our initial experiments were performed to test directly whether the 23-amino-acid Vpr interactor peptide, referred to as the dWF domain, could import IN into HIV-1 virions and restore integration to mature particles that would otherwise lack this function. This premise was based on previous studies from our laboratory demonstrating that the CAT gene product could be delivered into HIV-1 virions as a fusion to the dWF motif when the chimera was expressed *in trans* of an HIV-1 proviral expression clone (BouHamdan *et al.,* 1998). In order to assess whether the dWF could similarly transport an HIV-1 viral gene product, such as IN, several proviral expression constructs were obtained. The key features of three HIV-1 constructs, which are incapable of integrating HIV-1 DNA after reverse transcription, are shown in Fig. 1. The first construct tested bears a single mutation, D116N, an IN active site point mutation, and the two additional proviral clones bear stop codons within the coding sequence of IN that result in premature termination of the protein at amino acid positions 4 and 234. These plasmids are required in the complementation studies described below. Their use relies on the premise that IN is transported into HIV-1 virions by *in trans* expression of IN as a fusion to either Vpr or dWF. The constructs illustrated in Fig. 1B represent the expression cassettes for the chimeras, which are incorporated into virus particles. These IN fusions are next shown to be capable of catalyzing integration of viral DNA.

Figure 2 illustrates the results of a syncytia-forming assay from an infection of MAGI cells with three virus-containing supernatants prepared by single or cotransfection of selected expression plasmids described above. The approach is based on the expectation that cells bearing an integrated HIV-1 provirus would, over time, produce sufficient levels of the envelope gene product to induce multicellular fusion and Tat to trigger overexpression of β-galactosidase, which would stain infected cells deep blue after exposure to X-gal (Kimpton and Emerman, 1992).

As shown in Fig. 2A, MAGI cells infected with virions bearing the IN(D116N) mutation yield, almost exclusively, infrequent, single cells that are stained light to medium blue. In contrast, wild-type NL_{4-3} virions induce many large multinucleated deep blue syncytia, an example of which is shown in Fig. 2B. HIV-1 virions derived from cotransfection of 293T cells with pNL_{4-3}/IN(D116N) and SLX-CMV-dWF-PR-IN (Fig. 2C) also are capable of initiating syncytia formation, but to a significantly lesser extent, and generally do not involve the fusion of as many cells as observed with infection of the MAGI cells using wild-type HIV-1 particles. This seems appropriate since virions generated by cotransfection are expected to be capable of only a single round of infection, thereby limiting the overall population of cells capable of participating in syncytia formation. With regard to overall number, there were approximately four- to sixfold less syncytia for complemented virions versus infection with wild-type virus depending on the state of the MAGI cells since they may elicit a high background of singly stained cells or conversely be unresponsive to staining even after successful infection (see Discussion). Nevertheless, these

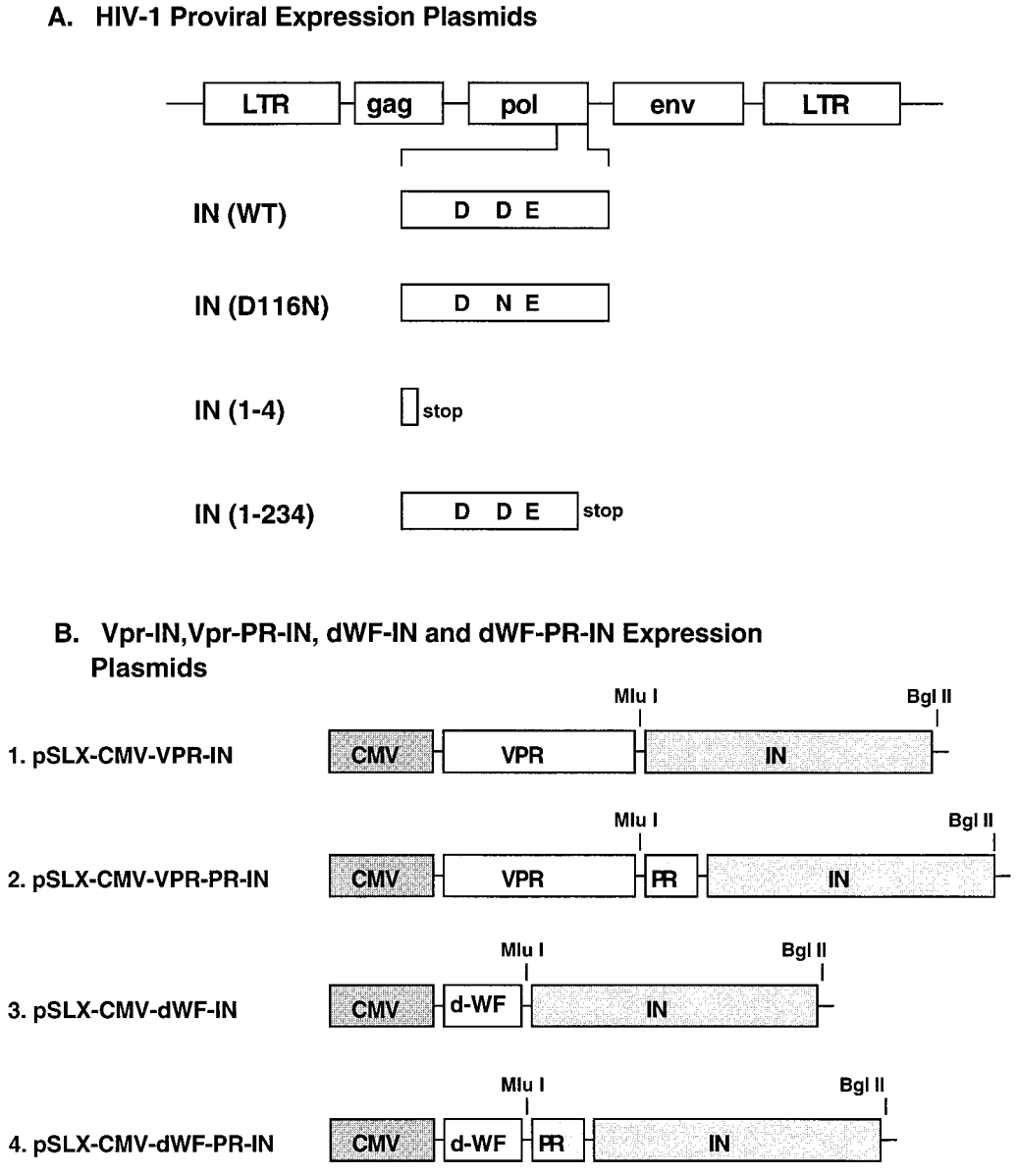

FIG. 1. Schematic of HIV-1 proviral DNA constructs and Vpr or dWF IN chimera expression plasmids. (A) The simple linear map of HIV-1 proviral DNA is shown (without accessory genes) highlighting the approximate region that encodes IN at the 3′ end of the *pol* domain whose integrity is required for integration of viral DNA. Below is shown the type of IN mutation in three HIV-1 proviral constructs, D116N, an active site mutant in the D,D(35)E domain (1), and two premature IN termination mutants, IN (1-4) and IN (1-234) (see Engelman *et al.,* 1995). All three plasmids produce virions that lack the ability to integrate HIV-1 DNA. PR designates the protease cleavage site that occurs between RNase H and IN and not HIV-1 protease itself.

initial experiments provided a clear indication that IN was incorporated into mature HIV-1 particles as an independently expressed fusion to the dWF domain and retained those functions required for the integration of HIV-1 DNA.

Import of the dWF-IN chimera is Vpr dependent

Next, we sought to demonstrate the actual presence of the dWF-IN chimera within virions and also determine whether delivery of the protein was dependent upon the coexpression of Vpr, as had been shown previously for fusion of dWxxF to the CAT gene product (BouHamdan *et al.,* 1998). dWF-IN, which lacks the protease cleavage site, was used since this species would exhibit slightly slower mobility in PAGE and by immunoblotting would appear as a band about 2.5 kDa larger than IN derived from natural protease processing of the HIV-1 Gag-Pol polyprotein precursor. As shown in Fig. 3, lane 1, infection with HIV-1 NL_{4-3} IN(D116N) alone shows a single discrete band at 32 kDa corresponding to IN(D116N). Virions derived from cells cotransfected with NL_{4-3} IN(D116N) and SLX-CMV-dWF-IN reveal the presence of

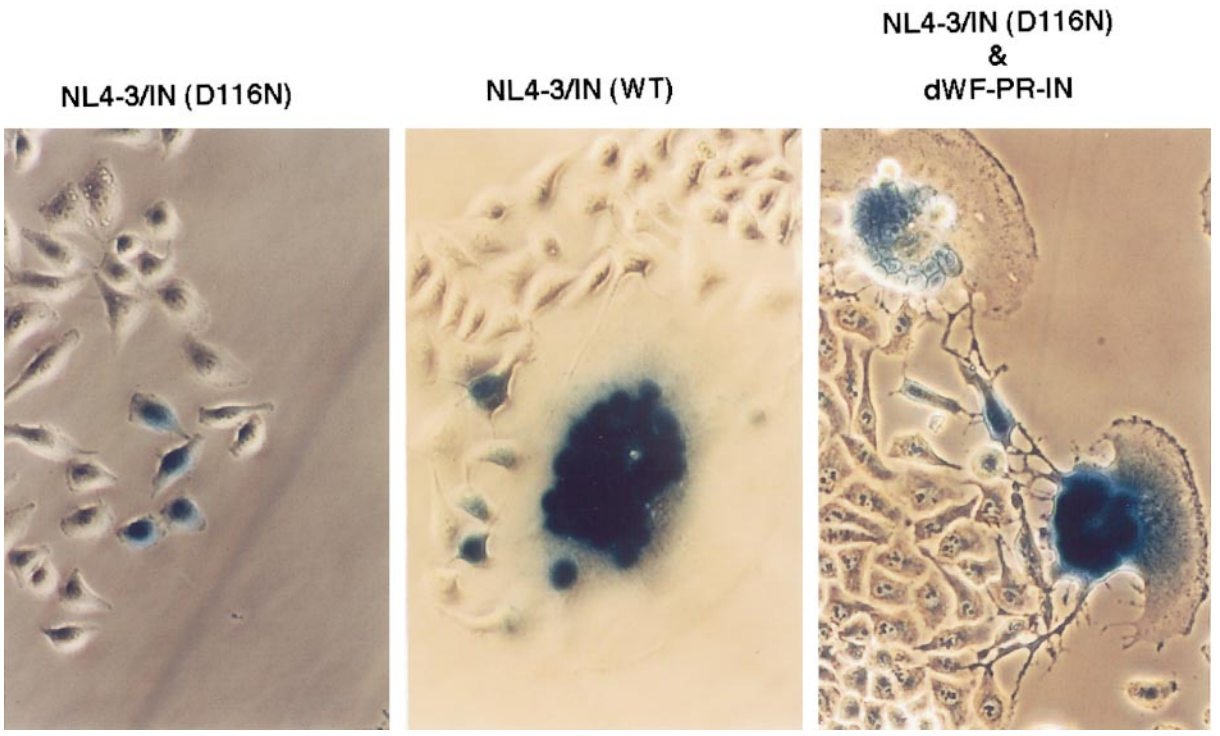

FIG. 2. dWF-PR-IN restores integration activity to HIV-1 IN (D116N)-defective virions as assessed by a syncytia-forming assay. HeLa $CD4^+$; β-gal (MAGI) cells were infected with virions derived from transfection of 293T cells with (left). pNL_{4-3}/IN (D116N) DNA alone; (middle). pNL4-3 DNA alone; (right) pNL_{4-3}/IN (D116N) DNA and SLX-CMV-dWF-PR-IN DNA. Cells were stained for β-galactosidase activity 3 days after infection.

two bands, as shown in Fig. 3, lane 2, IN (D116N) identical to that observed in lane 1, and also a less intense species slightly above IN(D116N) whose size is consistent with that predicted for dWF-IN. This protein is not present in virions derived from cotransfection of NL_{4-3} ΔVPR and SLX-CMV-dWF-IN (lane 3), even though IN derived from the proviral template at 32 kDa is nearly of equal intensity relative to that observed in lane 2. Analysis of particles from cotransfection of NL_{4-3} and SLX-CMV-dWF-IN, in lane 4, shows two bands of almost equal intensity, consistent with packaging of IN from natural Gag-Pol polyprotein processing and the slower migrating dWF-IN. This suggests that packaging of the dWF-IN chimera may be more efficient within wild-type particles than within particles bearing Vpr or IN mutations. This might be expected as aberrant infectivity and morphology have been observed previously for virions that bear a variety of IN mutations (Engleman *et al.,* 1995). It is also worth noting that the copy number of the chimeric protein imported into virions is dependent upon the ratio during

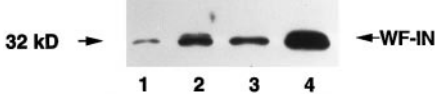

FIG. 3. Western blotting analysis of dWF-IN import into HIV-1 Vpr^+ and Vpr^- virions. 293T cells were transfected with: (1) pNL_{4-3}/IN (D116N) alone; (2) pNL_{4-3}/IN (D116N) + SLX-CMV-dWF-IN; (3) pNL_{4-3} (ΔVpr) + SLX-CMV-dWF-IN; and (4) pNL4-3 + SLX-CMV-dWF-IN. After transfection, supernatants from cells were harvested and virions were concentrated by ultracentrifugation through a 20% sucrose cushion. Virions were then subjected to PAGE/Western blot analysis using a polyclonal antiserum against HIV-1 IN. The slightly slower species visible above IN at 32 kDa represents the dWF-IN chimeric protein.

TABLE 1

Analysis of VPR-PR-IN, dWF-IN, and dWF-PR-IN Complemented IN-Defective HIV-1 Particles

HIV-1 Proviral template and IN fusion complement	No. of blue syncytia	% WT
D116N only	14	6.9
D116N + dWF-IN	42	20.8
D116N + dWF-PR-IN	52	25.7
D116N + Vpr-PR-IN	43	21.3
IN (1-234) only	13	6.4
IN (1-234) + dWF-PR-IN	44	21.7
NL4-3 (WT)	202	100.0

Note. Three HIV-1 expression plasmids (strain NL4-3), wild-type and two bearing IN mutations, were transfected into 293T cells alone or cotransfected with three expression plasmids bearing wild-type IN fused to Vpr or the dWF domain. PR represents the natural HIV-1 protease RT-IN cleavage site included between the fusion with Vpr and dWF. Viral supernatants derived from transfection 3 days later were normalized for p24 antigen by ELISA (each ~10 ng/ml) before application to MAGI reporter cells in 60 × 15 plastic culture dishes. Only deep blue syncytia were counted and the numbers above represent the average from duplicate plates. WT, wild-type.

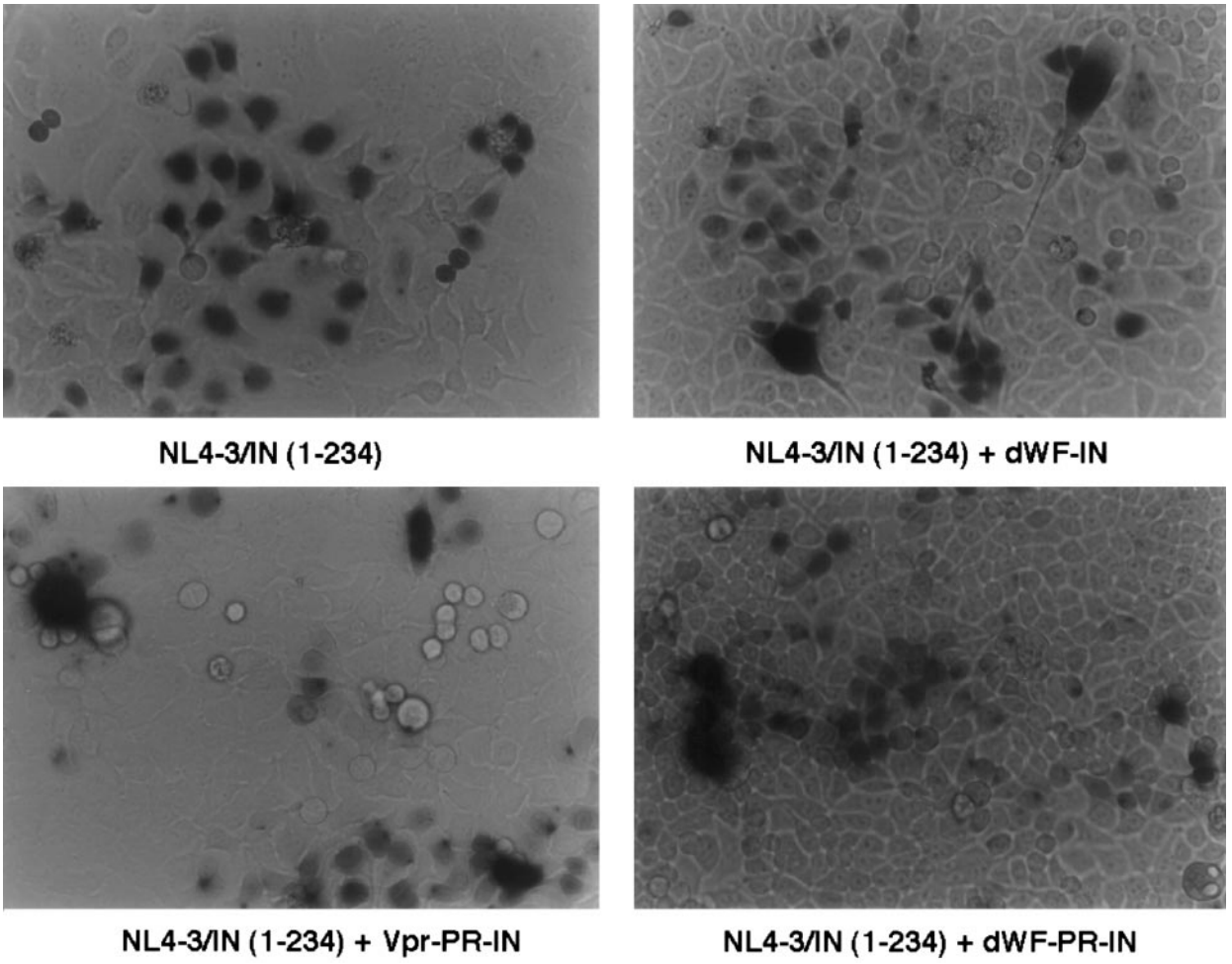

FIG. 4. Syncytia-forming assay illustrating complementation of pNL_{4-3}/IN (1-234) by various Vpr-IN or dWF-IN expression plasmids. MAGI cells were infected with virions derived from transfection of 293T cells with the DNA templates as indicated above each panel. Cells were stained for β-galactosidase expression 2.5 days later and representative fields of stained cells are shown.

transfection of the chimera expression plasmid relative to that of the proviral clone (Liu *et al.,* 1997; Fletcher *et al.,* 1997). In the comparisons of the complementation assays described below this molar ratio was generally two- to threefold higher with the Vpr or dWF-IN expression plasmids during transfections of the 293T producer cells to generate virus particles.

Comparison of the complementation efficiencies of HIV-1 IN-deficient proviruses by Vpr-IN and dWF-IN chimera expression plasmids

The relative efficiencies of the Vpr-IN and WxxF-IN chimera expression plasmids were tested in the MAGI assay for their ability to complement three IN mutant proviral clones. As shown in Fig. 1, the three integration-deficient proviruses included an IN active-site point mutant (IN D116N) and two premature IN termination mutants (IN 1-4 and IN 1-234). HIV-1 IN is normally 288 amino acids in length.

Based on the formation of deep blue syncytia, VPR-PR-IN, dWF-IN, and dWF-PR-IN appeared to complement all three mutants with equivalent efficiencies. As shown in Table 1, the noncomplemented IN (D116N) provirus yielded only a few "syncytia," approximately fourfold less than its complemented counterparts. These multiple blue cells scored as syncytia likely represent the background due to the division of a singly stained cell yielding a four-cell figure but nonetheless were included in the counting procedure. Figures 2 and 4 illustrate the distinct morphology of the deeply stained syncytia derived from infection with complemented virions. As shown in Table 1, virus from HIV-1 IN (D116N) cotransfected with IN chimera expression plasmids yielded syncytia that were approximately fivefold less in number than infection with wild-type HIV-1. This result concurs with that reported for complementation by VPR-PR-IN using different methods of analysis where the complemented virus was approximately 20–25% that of wild-type HIV-1 infection (Liu *et al.,* 1997; Fletcher *et al.,* 1997). This suggests that the MAGI assay based on syncytia formation was a reliable

TABLE 2

Analysis of VPR-PR-IN, dWF-IN, and dWF-PR-IN Complementation of IN Deletion Mutant HIV-1 Particles

HIV-1 Proviral template and IN fusion complement	No. of blue syncytia
IN (1-4) only	2
IN (1-4) + dWF-IN	10
IN (1-4) + dWF-PR-IN	18
IN (1-4) + Vpr-PR-IN	16
IN (1-234) only	3
IN (1-234) + dWF-IN	14
IN (1-234) + dWF-PR-IN	20
IN (1-234) + Vpr-PR-IN	18

Note. Experiments were performed as described in Table 1, except that MAGI cells were stained for β-galactosidase expression 2.5 days postinfection with equilibrated viral supernatants.

means of quantitating the relative extent of complementation.

In Table 2, the relative complementation efficiencies of IN derived from cotransfection of IN chimera expression plasmids with integration-deficient proviruses [IN (1-4) and IN (1-234)] were determined. The results from this analysis were similar to those described above. The numbers of syncytia scored were fewer owing to the smaller culture dish format but nevertheless were almost equal in number for VPR-PR-IN, dWF-IN, and dWF-IN, suggesting equivalent levels of complementation.

The relative level of IN-chimera incorporation into complemented HIV-1 virions

To be further assured that the MAGI assay based on syncytia formation demonstrated complementation efficiency with reasonable accuracy, the level of incorporation into virions for the various IN fusions was assessed. In Fig. 5A, a comparison of VPR-IN and dWF-IN incorporation into HIV-1 IN (D116N) particles is illustrated. In lane 1, VPR-IN, which lacks a protease cleavage site, yields a band of ~46.5 kDa that migrates above the proviral-derived 32-kDa IN (D116N). As shown in this lane, the quantity of these two species within the sucrose cushion-derived virus particles appears equal. In lane 2, the wide band likely represents a doublet of both IN (D116N) below and dWF-IN above that is about 2.5 kDa larger. The 10–20% Tris–HCl gel system was inadequate for the separation of these two species despite prolonged electrophoresis. Figure 5B illustrates separation of IN(D116N) and dWF-IN using 10–20% Tris–tricine PAGE. As shown in lane 2, dWF-IN appears to be incorporated at approximately the same level as IN(D116N). These results indicate that the Vpr and dWF chimeric IN species can be incorporated into HIV-1 particles typically at levels similar to IN derived from the provirus. Furthermore, the carrier proteins Vpr and dWF do not appear to differ dramatically in the efficiency of transporting their fusion partner into virions.

DISCUSSION

The WxxF motif was discovered initially as an interactor with Vpr using peptide phage display methodology (BouHamdan *et al.,* 1998). The occurrence of this motif within cellular proteins that are known to associate with HIV-1 Vpr such as uracil DNA glycosylase (BouHamdan *et al.,* 1996) and TFIIB (Agostini *et al.,* 1996) fortifies the notion that the diverse effects Vpr can have upon cells (Emerman, 1996) may be related to its ability to interact with additional, multiple cellular targets that also bear the WxxF motif (BouHamdan *et al.,* 1998). In an effort to exploit the interaction of the WxxF motif with Vpr, it was shown that a dimer of the WxxF motif fused to the CAT gene resulted in efficient incorporation of the chimeric protein into sucrose gradient purified HIV-1 virions (BouHamdan *et al.,* 1998). Additional studies comparing the transport of the CAT gene product into virus particles as a fusion to Vpr or dWF revealed comparable levels of incorporation, based upon the quantitation of virion-associated CAT activity. As expected, transport of CAT via fusion to the dWF motif was dependent upon expression of Vpr from the proviral template (BouHamdan *et al.,* 1998).

The initial and elegant efforts of incorporating a variety of proteins as fusions to Vpr provided the first means to introduce proteins into HIV-1 particles at reasonable levels that were not dependent upon direct expression from the proviral clone (Liu *et al.,* 1997; Fletcher *et al.,* 1997). Interestingly, the ability of the Vpr fusion partner to manifest its expected activity varied, however. For instance, direct fusion of HIV-1 reverse transcriptase (RT) to Vpr does not hamper the chimera's ability to complement RT-deficient virus, unlike a similar fusion context to HIV-1 IN. In this case, introduction of an intervening HIV-1 protease site between Vpr and IN is necessary for complementation of IN mutant proviral clones (Liu *et al.,* 1997; Fletcher *et al.,* 1997). The release of IN from Vpr may be required for proper positioning within the HIV-1 nucleocapsid or the simple presence of the 96-amino-

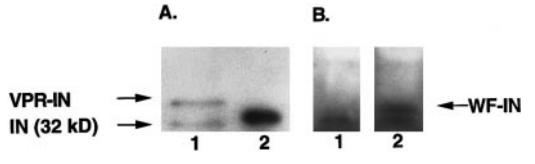

FIG. 5. Western blotting analysis of dWF-IN and Vpr-IN import into HIV-1 virus particles. HIV-1 virions were produced and isolated as outlined in Fig. 3 after transfection of 293T cells with (Lanes 1) pNL4-3/IN (D116N) + pSLX-CMV-VPR-IN or (Lanes 2) pNL4-3/IN (D116N) + pSLX-CMV-dWF-IN. (A) Virion proteins separated in a 10–20% Tris–HCl gradient gel. (B) Virions electrophoresed through a 10–20% Tris–tricine gradient gel.

acid Vpr at the N-terminus of IN may sterically occlude IN's function since multimerization of the protein is required for integration activity (Jones *et al.,* 1992; Van Gent *et al.,* 1993). A comparison among dWF-IN, dWF-PR-IN, and VPR-PR-IN using the MAGI cell assay suggests there is not likely to be more than a twofold difference, and in fact they appear to be equivalent in their ability to complement an IN-deficient proviral clone. These results concur with the analysis of CAT activity imported into HIV-1 virions by fusion to Vpr or dWF (BouHamdan *et al.,* 1998). This equivalency of activity was observed for the complementation of three IN-deficient proviral clones, one with an active-site point mutation, IN(D116N), and two premature termination mutants that truncate IN after the 4th amino acid, IN(1-4), and after the 234th amino acid, IN(1-234).

It is known that results from the MAGI cell assay can be difficult to assess (Engelman *et al.,* 1995). A relatively high background, especially of single light blue cells even prior to infection, and somewhat more deeply stained cells following infection with HIV-1 in the absence of integration occurs due to some production of low levels of Tat from unintegrated HIV-1 DNA (Stevenson *et al.,* 1990). Conversely, MAGI cells passaged for a prolonged period of time, particularly in the absence of growth in the appropriate selection medium, may stain poorly despite infection and integration of HIV-1 DNA. Due to these deficiencies, only deeply stained syncytia were counted to determine the relative efficiency of complementation among the IN chimeras and comparisons were made using identical batches of MAGI cells. Although other investigators most recently used a more sensitive and quantitative colony-forming assay to assess complementation efficiency, the use of this system is not feasible since the proviral clones lack functional Vpr (Liu *et al.,* 1997; Fletcher *et al.,* 1997).

Western blotting analyses to detect the incorporation of IN chimeras within virions purified through sucrose cushions yielded results that concur with the MAGI assay complementation data. IN coupled to Vpr or dWF, with or without an intervening protease cleavage site, was found to be present within HIV-1 viral particles. Although the Western blotting technique is not a rigorous quantitative procedure, the analysis of virions prepared similarly indicated that the chimeras were incorporated into virions at nearly equivalent levels and certainly within twofold of each other, as VPR-IN and dWF-IN seemed to be present at levels reasonably equivalent to IN derived from the HIV-1 IN (D116N) proviral template, which serves as an internal standard. Virions analyzed for the appearance of IN processed proteolytically from either Vpr or dWF also indicated perhaps less than twofold reduction of IN derived from fusion with dWF versus Vpr (data not shown). It seems that fusion of the 23-amino-acid dWF motif to IN versus the larger Vpr protein has the advantage of not having to be released from IN in order to catalyze the integration reaction since comparable levels of complementation and similar levels of incorporation into HIV-1 virions were observed.

Clearly, the transport of a variety of proteins into HIV-1 particles can be accomplished by direct fusion to either Vpr or the Vpr interactor domain, WxxF. The use of both systems will likely provide the means to initiate a variety of critical studies by allowing the direct delivery into particles of virion-associated proteins, apart from those encoded by the proviral DNA. Perhaps more interesting is the ability to assess the effect upon virion infectivity following the incorporation of protein-based molecular therapeutics into HIV-1 virions. Such studies, though in their infancy, have been initiated and the results will likely provide new insights into lentiviral particle formation, architecture, release, and infectivity.

MATERIALS AND METHODS

Plasmid construction

The HIV-1 molecular clones used include $pNL_{4\text{-}3}$ and $pNL_{4\text{-}3\Delta VPR}$, obtained from the AIDS Reagent Repository, NIH, as well as HIV-1 pNL4-3, which bear the IN active-site point mutation, D116N, or IN termination mutants IN(1-4) and IN(1-234), kindly provided by Dr. Alan Engelman at the Dana Farber Cancer Institute (Boston, MA). DNA fragments for insertion into pSLX-CMV-Vpr and pSLX-CMV-dWF expression vectors (BouHamdan *et al.,* 1998) were amplified by PCR from the bacterial expression plasmid, pKK223-NY5-IN, kindly provided by Parke-Davis Pharmaceuticals Inc., using Vent polymerase (New England Biolabs). The use of the IN expression cassette from pKK223-NY5-IN is important since the DNA sequences that encode IN have been changed significantly and are unable to recombine with the proviral-encoded DNA sequence. The 5′ primers for the amplification reactions contained a unique *Mlu*I site followed by sequences in-frame for IN or PR-IN. PR-IN incorporates sequences in the primer to append an additional 10 amino acids upstream of IN that add the natural HIV-1 protease cleavage site at the junction between RNase H and IN. The sequences of the forward primers are 5′-GCGCAAGCTTCGTTCCTGGACGGTATCGAT-3′ (without the protease cleavage site) and 5′-CCCACGCGTTTGGTCAGTGCTGGAATCAGGAAAGTACTATTCCTGGACGGT-3′ (with the protease cleavage site). The common 3′ primer for amplification of all inserts is anchored upon terminal IN sequences followed by a *Bgl*II site for insertion into the SLX-CMV vector whose sequence is 5′-GGCAGATCTAAGCTTTAGCTTCGTCCTG-3′. All plasmids were sequenced through the inserts for their integrity prior to use.

Cell transfection and infection

Using the calcium phosphate method for delivery of DNA into cells (Profection, Promega), the human kidney

carcinoma cell line 293T was transfected alone with HIV-1 proviral clones as indicated or cotransfected with these templates and SLX-CMV-VPR-IN, SLX-CMV-VPR-PR-IN, SLX-CMV-WF-IN, or SLX-CMV-dWF-PR-IN, the quantity of which is as indicated in the figure legends. Three days posttransfection cell supernatants containing virus were harvested and clarified by low-speed centrifugation. HeLa-CD4-LTR/β-gal cells grown in either 60 $\times$ 15 or 35 $\times$ 10 mm plates were infected in duplicate at $\sim$10–15% confluency with various viral stocks. Addition of supernatant to these MAGI reporter cells (Kimpton and Emerman, 1992) was adjusted for each culture based upon HIV-1 p24 antigen content as quantitated by ELISA analysis. MAGI cells contain an integrated cassette consisting of the the β-galactoside gene (β-gal) under the control of the HIV-1 long terminal repeat and therefore can be stained for β-galactosidase activity typically 48–72 h postinfection as indicated in the figure legends. The infected cells were then fixed with a solution of 0.2% glutaraldehyde and 1% formaldehyde for 5 min at room temperature. After fixation, the cells were washed with phosphate-buffered saline and stained with bromo-4-chloro-3-indolyl-β-D-galactopyranoside for 2 h at 37°C. Representative fields of stained cells were photographed and the numbers of deeply stained syncytia were determined either by visual counting through a microscope or from series of photographs taken in various fields of the stained cells using an Olympus CK2 microscope.

Western blotting analyses of complemented HIV-1 virions

293T cells were grown in duplicate 100 $\times$ 20 mm culture dishes and transfected as described in the previous section. Virions released into the cell culture medium 2.5–3 days posttransfection were centrifuged at 2000 rpm for 5 min at 4°C to remove cellular debris. Afterward, virions were concentrated by adding 10 ml of cell medium onto 2 ml of buffered, isotonic 20% sucrose and the cushioned medium was ultracentrifuged at 40,000 rpm for 2 h. The bulk of the solution was removed by careful pipeting, and the pelleted virions visible as slight white pellets were removed from the bottom of the ultracentrifuge tubes by resuspension in residual medium, typically resulting in a volume of 50 to 100 μl of concentrated virus particles. The maximal volume of particles was loaded into 10–20% Tris–HCl or Tris–tricine precast gradient gels (Bio-Rad) and electrophoresed until the bromophenol blue marker migrated to at least the bottom of the gel. Proteins from the gel were transferred to a Polyscreen transfer membrane using a semidry transfer apparatus as recommended by NEN Life Science Products. IN or IN chimeric proteins were detected using a rabbit anti-IN polyclonal serum (kindly provided by Dr. Anna Marie Skalka, Fox Chase Cancer Center, Philadelphia, PA) and visualized by chemiluminescence development (NEN Life Science Products) of bound HRP-conjugated anti-rabbit IgG antisera used at 1/1500 dilution (Sigma).

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